

## STATE-OF-THE-ART REVIEW

**Targeting invasive properties of melanoma cells**Imanol Arozarena<sup>1</sup> and Claudia Wellbrock<sup>2</sup><sup>1</sup> Cancer Signalling Group, Navarrabiomed (Miguel Servet Foundation), Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain<sup>2</sup> Manchester Cancer Research Centre, Faculty of Biology, Medicine and Health, The University of Manchester, UK**Keywords**co-operative invasion; invasion; melanoma; MITF; PDE5A; phenotype switching; RAC; RHO; TGF $\beta$ ; WNT5A**Correspondence**

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Melanoma is a skin cancer notorious for its metastatic potential. As an initial step of the metastatic cascade, melanoma cells part from the primary tumour and invade the surrounding tissue, which is crucial for their dissemination and the formation of distant secondary tumours. Over the last two decades, our understanding of both, general and melanoma specific mechanisms of invasion has significantly improved, but to date no efficient therapeutic strategy tackling the invasive properties of melanoma cells has reached the clinic. In this review, we assess the major contributions towards the understanding of the molecular biology of melanoma cell invasion with a focus on melanoma specific traits. These traits are based on the neural crest origin of melanoma cells and explain their intrinsic invasive nature. A particular emphasis is given not only to lineage specific signalling mediated by TGF $\beta$ , and noncanonical and canonical WNT signalling, but also to the role of PDE5A and RHO-GTPases in modulating modes of melanoma cell invasion. We discuss existing caveats in the current understanding of the metastatic properties of melanoma cells, as well as the relevance of the 'phenotype switch' model and 'co-operativity' between different phenotypes in heterogeneous tumours. At the centre of these phenotypes is the lineage commitment factor microphthalmia-associated transcription factor, one of the most crucial regulators of the balance between de-differentiation (neural crest specific gene expression) and differentiation (melanocyte specific gene expression) that defines invasive and noninvasive melanoma cell phenotypes. Finally, we provide insight into the current evidence linking resistance to targeted therapies to invasive properties of melanoma cells.

**Introduction**

Cutaneous melanoma accounts for only < 5% of all common skin cancers, yet it causes the majority of skin cancer deaths [1]. One of the main reasons for the lethality of melanoma is its metastatic propensity, which is partly reflected in the aggressive invasion of melanoma cells into neighbouring tissue at a time when the primary tumour is still significantly small in size. The invasive behaviour of melanoma cells appears to be a remnant of their neural crest origin; a trait that

distinguishes this cancer from the other nonmelanoma epithelial derived skin cancers. Because of their invasive potential, melanoma cells have been extensively used to study general mechanisms of cancer cell invasion [2–5]. Moreover, the idea of targeting melanoma cell invasion as means of therapeutic intervention stimulated an era of intense research with the aim of discovering the main regulator(s) of melanoma invasiveness.

**Abbreviations**

ECM, extracellular matrix; EMT, epithelial mesenchymal transition; HGF, hepatocyte growth factor; MAPK, mitogen activated protein kinase; MITF, microphthalmia-associated transcription factor; MMP, matrix-metalloprotease; RGP, radial growth phase; TGF $\beta$ , transforming growth factor beta; VGP, vertical growth phase; W-RAMP, WNT-mediated receptor-actin-myosin polarity.

During the last years, the identification and specific targeting of genetic drivers of melanoma cell proliferation and survival, such as BRAF and other activators of the mitogen activated protein kinase (MAPK) pathway, along with the recent development and successes of immunotherapy has taken away the attention from 'invasiveness' as a crucial target for melanoma therapy. However, no therapy is unflawed, and patients who relapse with acquired resistance to BRAF and MEK inhibitors often present with melanomas that display a much more aggressive and invasive phenotype [6,7]. Furthermore, not every patient responds to immunotherapy and again the phenotypes linked to innate resistance contain signatures linked to the invasive phenotype [8]. Hence, because invasive properties play important roles at every step of melanoma development, and because an invasive phenotype appears to be linked to therapy resistance, there might still be a place for targeting invasive properties in melanoma.

### **Melanoma cells, melanocytes and the neural crest**

Cutaneous melanoma is a cancer of transformed epidermal melanocytes, pigment cells that originate from the neural crest [9]. During development, the expression of the microphthalmia transcription factor (MITF) commits neural crest cells to the melanocyte lineage and marks melanoblasts in the dorsolateral neural crest migration pathway [10]. These melanoblasts are highly motile, migrate throughout the embryo and colonise the basal layer within the epidermis, where they eventually differentiate into mature melanocytes. Postmigratory melanocytes are attached to the extracellular matrix (ECM) of the basement membrane of the epidermis and they exist in a homeostatic relationship with epidermal keratinocytes. Nevertheless, melanocytes still display a motile behaviour, although this is very much controlled by the neighbouring keratinocytes to which they closely adhere via cadherins, connexins and other adhesion receptors [11,12]. However, this control is lost in melanoma cells, allowing the migratory programme to be fully reactivated.

### **Dermal invasion, extravascular migration and EMT**

At the early stages of melanoma development, transformed melanocytes display uncontrolled proliferation within the epidermis [radial growth phase (RGP)], giving rise to melanoma *in situ* [11]. While melanoma *in situ* is not invasive, RGP melanoma cells are highly

susceptible to molecular changes and microenvironment derived signals that can stimulate their invasive properties and induce the vascular growth phase (VGP). In this context, the interactions with keratinocytes play a major role, and as mentioned earlier, loss of these interactions supports detachment and invasion [13]. Moreover, keratinocytes can contribute to dermal invasion of melanoma cells; they produce hepatocyte growth factor (HGF), which can downregulate E-cadherin [14], secrete matrix-metalloproteinase 9 (MMP-9), which helps breaking down the basement membrane [15] and they can activate Notch signalling which induces invasion by upregulation of miR-222/221 [16].

Invasion into dermal tissue allows intravasation and dissemination through the vascular route. However, melanoma also displays lymphatic invasion and angiotropism, which can be seen as extravascular migration [17]. Lymphatic invasion has been detected in ~16–47% of invasive melanomas [18], and the occurrence of angiotropism has been reported in up to 70% of cases and is suggested as an independent prognostic marker predicting risk for metastasis [19,20]. However, due to the lack of precise markers that could be used in routine analyses, so far no larger study has been conducted to assess the incidence of angiotropism [18]. Angiotropic melanoma cells migrate in a pericyte-like manner (pericytic mimicry) along extracellular surfaces of the vasculature without intravasating. Intriguingly, such behaviour is also observed during early neural crest cell/melanoblast migration. Little is known about the molecular players involved in extravascular migration, but angiotropic melanoma cells display a gene signature including neural precursor markers and regulators of migration such as *CCL2*, *ICAM1*, *IL6*, *SERPINE2* and *PDGFR* [21]. A similar gene signature is induced by TNF $\alpha$ , which in fact can stimulate angiotropism in the context of UV-induced inflammation [22]. This observation provides a logical and important link between inflammation and melanoma cell dissemination [20].

The different gene signature features of angiotropic melanoma cells also reveal a very important aspect of melanoma cell motility. As mentioned above, melanoma cells are not of epithelial origin but derived from highly motile neural crest cells, which had undergone 'epithelial mesenchymal transition' (EMT) while leaving the neural tube. As a consequence, and despite specific differentiation at their final destination, epidermal melanocytes still express several EMT markers, such as vimentin, N-cadherin, ZEB2 and SLUG, a property which is thought to predispose them to metastasis once they transform [23,24]. Thus, when

melanoma cells become invasive, they do not undergo a 'classical' EMT comparable to what is seen in epithelial cancers; rather it appears that de-differentiation towards their neural crest origin is required for motility [25]. Importantly, however, while the requirement for de-differentiation is supported by many observations, what really defines the 'de-differentiated state' is less clear, i.e. is the 'neural crest' (MITF negative) state required or is the 'melanoblast' (MITF positive) state sufficient? After all, it is the melanoblast and not the neural crest cell that performs long distance migration in the embryo. No thorough comparable study has addressed this important question, but an answer would certainly help to identify the markers crucial for melanoma cell invasion without distraction by putative melanoma 'stem cell' markers.

### Specific drivers of melanoma cell motility and invasion

General regulators of the actin cytoskeleton that play a role in cancer cell motility and invasion, such as SRC and FAK, are also relevant in melanoma and various studies have shown that inhibition of these kinases will reduce melanoma cell invasion [26–29]. However, while the broad-spectrum tyrosine kinase inhibitor dasatinib, which also inhibits SRC kinases, is effective in melanoma cells in preclinical studies [30], a phase II trial revealed that dose-limiting toxicity is a major obstacle [31], which dampened the enthusiasm for targeting such general regulators, and stimulated research into more specific features of melanoma cell motility.

The chase for melanoma specific regulators of migration and invasion led to the identification of various factors and signalling events that control melanocyte lineage commitment and migration in early development. Among them are canonical WNTs (e.g. WNT3), transforming growth factor beta (TGF $\beta$ ) or noncanonical WNTs (e.g. WNT5A). Importantly, these factors not only directly impact on invasive behaviour by regulating the actin cytoskeleton, but they also initiate cellular signalling that ultimately controls the expression levels and the function of the lineage commitment factor MITF [32].

### The complex role of MITF in melanoma cell invasion

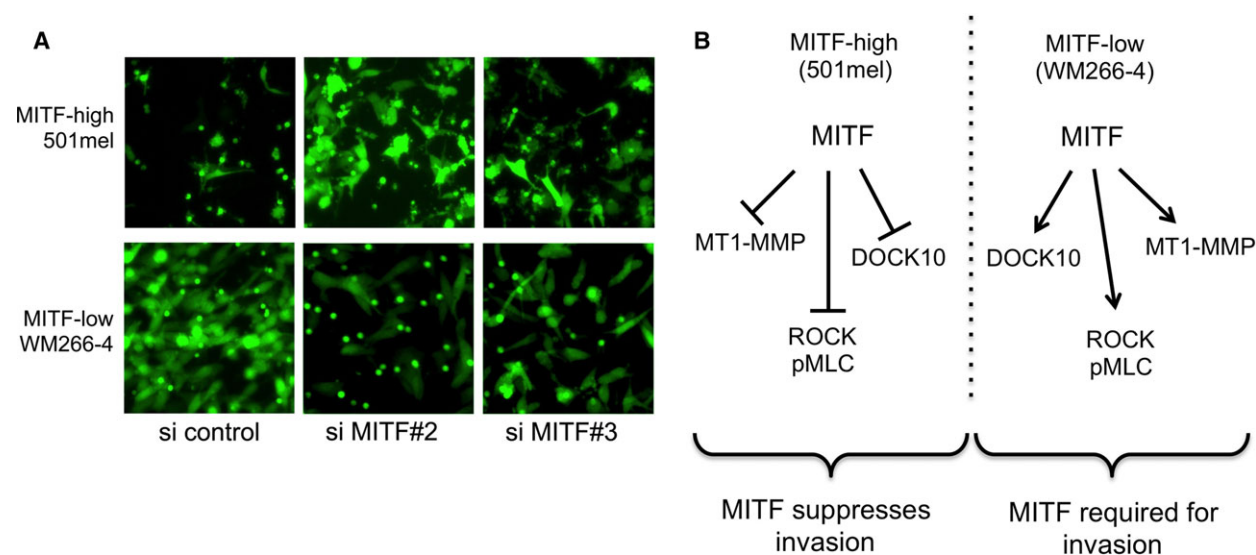
Microphthalmia-associated transcription factor is thought to be one of the most crucial regulators of the balance between differentiation (melanocyte specific gene expression) and de-differentiation (neural crest

specific gene expression). However, MITF's role in invasion is by far not clear.

It is assumed that MITF while inducing differentiation is a suppressor of invasion, and this is based on three facts: First, it is well established that gene expression profiling of melanoma cells confidently identifies a highly 'invasive phenotype' characterised by extremely low MITF expression, and consequently, a signature of melanocyte de-differentiation linked to markers of neural crest, EMT and stemness [33]. The idea is that the phenotype linked to this signature possesses properties of neural crest cells, which explains the increased motility that these cells display. Secondly, and in line with the above, factors that reduce MITF expression, e.g. WNT5A, TGF $\beta$  or hypoxia [34–36] also increase the invasive potential of melanoma cells [37–39]. Importantly, genes like *WNT5A*, *TGF $\beta$ beta*, their related signatures and a hypoxia signature are all included in the 'invasive/MITF-low' signature [33]. Finally, the cell line 501mel, a cell line abundantly used in the field of 'MITF-research', expresses extremely high levels of MITF (due to a *MITF* gene amplification and presence of mutated beta-catenin) and is poorly invasive. Moreover, MITF depletion from 501mel cells leads to increased invasion [40–42] and see Fig. 1A. Similar results are found with the high-MITF expressing noninvasive melanoma cells lines WM3682 and WM3526 [43] or the high-MITF expressing mouse cell line B16 [34]. Likewise, increasing MITF expression in 'invasive/MITF-low' melanoma cells (WM1716, WM3314 or WM266-4) suppresses invasion [40,43].

While all the above facts seem to settle the case for MITF being a suppressor of invasion, so far nobody has examined whether MITF is actually required for invasion in the 'invasive/MITF-low' cells. This could in fact be the case as not only neural crest cells are motile, but also melanoblasts, which do express MITF [10]. Indeed, we found that depletion of MITF in low MITF expressing highly invasive WM266-4 melanoma cells leads to a dramatic decrease in invasion (Fig. 1A). Thus, there might be a proinvasive role for MITF after all. Indeed, MITF regulates the expression of a large set of genes that are linked to the GO terms 'actin cytoskeleton', 'migration' and 'invasion', and while in cell lines expressing high levels of MITF it suppresses the expression of these genes, in cell lines with low MITF expression levels, it actually induces them (Fig. 1B and Z. Miskolczi, C. Wellbrock, unpublished data).

A proinvasive role for MITF is also supported by the fact that the receptor c-MET that mediates HGF stimulated melanoma cell invasion is a MITF target gene [44,45], and that ectopic MITF overexpression



**Fig. 1.** The effects of reducing MITF expression levels on melanoma cell invasion. (A) RNAi mediated reduction in MITF expression increases invasion of MITF-high 501mel cells and reduces invasion of MITF-low WM266-4 cells into 3D dermal collagen. Both cell lines express GFP and have been analysed using FluoroBlok inserts coated with collagen gels. (B) Model indicating the opposite function of MITF in MITF-high and MITF-low cells.

increases 501mel cell invasion in response to HGF [45]. The ‘melanoma predisposition’ mutant MITF<sup>E316K</sup> also enhances 501mel invasion [46]. Finally, many cell lines that are considered to belong to the MITF-expressing (MITF-high) group display invasive behaviour in 3D extracellular matrix (ECM) systems.

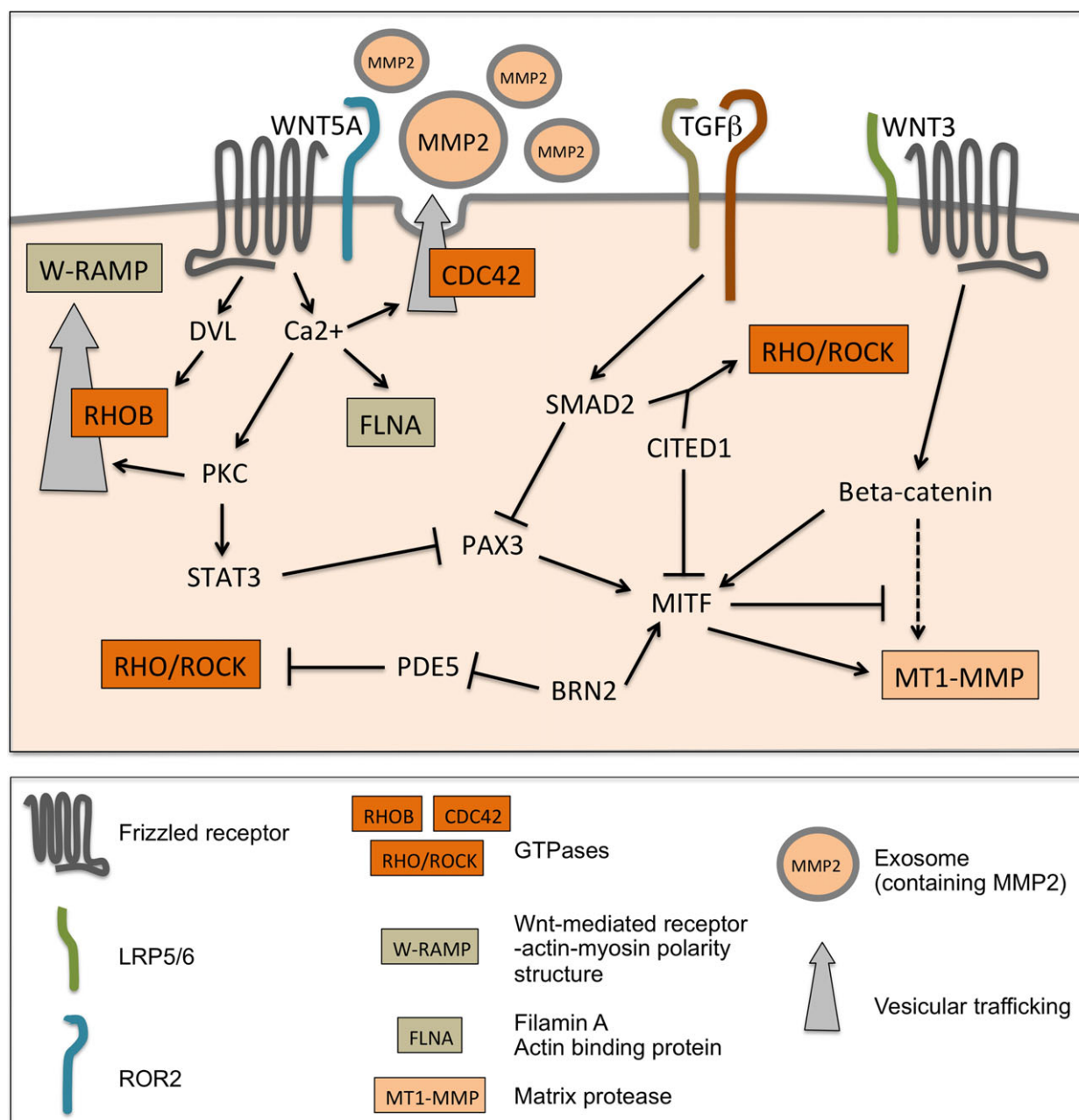
In summary, the role of MITF in invasion appears to be more complex than generally assumed, and ‘invasive’ signatures linked to low MITF expression as well as the fact that factors that induce invasion also downregulate MITF are muddying the water. In an era of ‘omics’, profiling signatures are surely very helpful, but the case of MITF highlights that the relationship between ‘signature’ and functional behaviour might not always be as simple as assumed. It seems to fully comprehend MITF’s role in invasion its expression and function has to be seen in context of each particular signalling network.

### The Yin and Yang of noncanonical and canonical Wnt signalling

One such signalling network is downstream of non-canonical WNT with WNT5A being a major player [47]. In conjunction with ROR2, WNT5A binds to frizzled receptors and drives invasion through intracellular  $\text{Ca}^{2+}$  and protein kinase C (PKC) [38,48] (Fig. 2). WNT5A can control directional movement by activating localised  $\text{Ca}^{2+}$ -induced actin myosin contraction. This occurs through RHOB and the Wnt-

mediated receptor-actin-myosin polarity (W-RAMP) structure, which contains actin, myosin IIB and melanoma cell adhesion molecule (MCAM) [49,50]. Furthermore, WNT5A induced  $\text{Ca}^{2+}$  signalling can stimulate calpain1-mediated cleavage of the actin cross-linker filamin-A [51]. Thus, WNT5A has a major impact on actin cytoskeleton dynamics. Moreover, a role for WNT5A in vesicular trafficking in melanoma cells is also seen in the CDC42-dependent release of exosomes, which amongst other proteins also contain MMP2 [52], suggesting that WNT5A also contributes to the remodelling of the ECM.

In addition to its role in actin and ECM dynamics, WNT5A induces expression of vimentin and SNAIL and suppresses PAX3, a transcriptional regulator of MITF, and thereby reduces the expression of melanoma differentiation genes [35] (Fig. 2). The ‘Yang’ to noncanonical Wnt signalling in melanoma is canonical Wnt signalling (Fig. 2), which is required for early melanocyte lineage commitment and differentiation by inducing beta-catenin-mediated expression of MITF [53]. Beta-catenin mutations in melanoma are rare, and nuclear beta-catenin expression has been linked with good prognosis [54,55]. Moreover, mutated/stabilised beta-catenin induces high MITF expression, and MITF blocks the proinvasive activity of beta-catenin [40]. In line with this, overexpression of a stabilised beta-catenin mutant in the melanocyte lineage of mice in which melanoma development is either driven by NRAS or BRAF<sup>V600E</sup>/Pten results in primary



**Fig. 2.** Signalling that activates melanoma cell motility and invasion. TGFβ stimulates the activation of SMAD2, which together with the cofactor CITED1 induces the expression of genes that regulate RHO/ROCK mediated contractility and invasion [67]. WNT5A can directly regulate the actin cytoskeleton and hence motility through calpain-mediated cleavage of filamin A (FLNA) [51], and regulate invasion through the induction of various genes downstream of PKC [48]. WNT5A also inhibits canonical WNT3A signalling, which otherwise suppresses invasion partly by inhibiting MT-MMP expression [40]. WNT5A also regulates vesicular trafficking and thus contributes to the release of MMP2 containing exosomes [52] as well as to the localisation of the W-RAMP structure to the edge of the cell [49,50]. Downstream of TGFβ SMAD2 also suppresses PAX3, a transcriptional regulator of MITF [104], and CITED1 can also suppress MITF [65]. PAX3, and consequently MITF are also suppressed by WNT5A through the activation of STAT3 [35], however, BRN2 which is a positive regulator of invasion by suppressing the RHO inhibitor PDE5A [60] induces MITF expression [107].

tumours with a low degree of invasiveness and high degree of pigmentation, an indicator of differentiation [56,57]. Intriguingly, however, the presence of

stabilised beta-catenin in these mice dramatically increases metastatic burden and the observed metastatic tumours are highly pigmented [56,57]. The exact

mechanisms underlying this phenomenon are so far unknown, but it suggests that reduced ability to invade does not necessarily preclude metastatic potential. Possibly, high proliferative activity or prevention of anti-tumour immunity, which both have been linked to beta-catenin mutations in melanoma [58,59] could also be a determinant for metastatic behaviour.

### **BRN2 controls PDE5A, a suppressor of melanoma cell invasion**

An important suppressor of melanoma cell invasion is the cGMP-specific phosphodiesterase PDE5A, which removes the cGMP required for  $\text{Ca}^{2+}$  triggered actin-myosin contractility and invasion [60]. In line with such a suppressor role, a follow-up prospective cohort study linked the use of the PDE5 inhibitor sildenafil with an increased risk of melanoma, a correlation still under debate [61]. As PDE5A is a negative regulator of invasion, its transcriptional suppression, which is executed by BRN2 (Fig. 2), is correlated with increased invasion [60]. This reveals BRN2 as a positive regulator of invasion, which is in agreement with the observation that expression from the *BRN2* promoter is increased in motile cells, when analysed *in vivo* by intravital imaging [62]. This study also assessed a potential relationship between the differentiation state (i.e. pigmentation) and BRN2 expression in motile cells, again with the idea that motile cells are more de-differentiated, and possibly BRN2 could contribute to this phenotype. Indeed, pigmentation was greatly downregulated in motile cells, however, this was not significantly correlated with the increase in activity from the BRN2 reporter [62], suggesting that reduced pigmentation and increased BRN2 expression are two independent events linked to invasion.

### **TGF $\beta$ and the 'invasive' phenotype**

Pinner and co-workers also found that TGF $\beta$  signalling suppressed pigmentation and induced migration [62]. This observation is in line with the fact that 'active TGF $\beta$  signalling' is a key determinant of the 'original' invasive/MITF-low signature, described by Hoek and co-workers [33]. In agreement with a role in de-differentiation, TGF $\beta$  can maintain the melanocyte stem cell state by directly suppressing the expression of PAX3 (and hence MITF, see Fig. 2) in melanocytes [63]. TGF $\beta$  can also suppress MITF expression through GLI2 [64] or through CITED1 [65]. So far, these observations are all in agreement with a simple model in which TGF $\beta$  suppresses MITF expression and drives cells towards the de-differentiated and invasive phenotype. However, several findings suggest that

this model is in fact not that simple and that possibly the de-differentiation (achieved through suppression of MITF) and the regulation of invasion are two independent activities downstream of TGF $\beta$ . For instance, we have shown that in melanoma cells TGF $\beta$  also suppresses PAX3 and consequently MITF [66], but we find that in many melanoma cell lines, despite efficiently downregulating MITF, TGF $\beta$  does not increase invasion (unpublished data). Furthermore, while manipulating GLI2 impacts on melanoma cell invasion, whether the suppression of MITF downstream of TGF $\beta$  is actually required for invasion to occur has not been shown [64]. In addition, despite being able to suppress MITF expression, CITED1 displays significant coexpression with MITF [65], and while this predicts that only low CITED1 expression is linked to the 'invasive' signature, high expression of a CITED1 specific gene signature [65] as well as CITED1 itself [67] is correlated with poor prognosis. This might be due to the fact that apart from suppressing MITF, CITED1 is actively involved in TGF $\beta$  stimulated melanoma cell invasion by inducing the transcription of genes encoding important actin cytoskeleton regulators, such as ARHGEF5 and MRIP, which regulate cortical actin myosin contraction [67]. Thus, while TGF $\beta$  induced signalling is clearly linked to invasion, whether the suppression of MITF and its target genes is required for its proinvasive activities is so far unclear.

### **The role of RHO-GTPases in melanoma invasion**

Although there is broad evidence on the key role of RHO/ROCK signalling in melanoma cell invasion, no activating mutations have been found in RHO in human melanoma. On the other hand, RAC activating mutations are present in 4–7% of human melanomas with the mutation P29S in RAC1 being the third most recurrent human melanoma mutation after BRAF<sup>V600E</sup> and NRAS<sup>Q61L</sup> [68,69]. *In vitro* studies show that while the P29S mutation leads to RAC1 GTPase hyperactivation and lamellipodia formation, it actually reduces RAC's ability to form invadopodia and modify the ECM [70], questioning that the P29S mutation contributes to melanoma development predominantly by acting on invasion. Studies in transgenic melanoma models have demonstrated that although active RAC cannot drive melanoma, it cooperates with oncogenic RAS to promote melanoma proliferation and invasion [71,72]. In line with these reports, the RAC-specific exchange factor PREX-1 is overexpressed in human melanoma and drives melanoma metastasis while

another member of the family, PREX-2 is found mutated in human melanoma, increasing its enzymatic activity towards RAC1 to drive gene expression and the cell cycle by activation of the PI3K pathway [73–75].

Because melanoma cells are extremely motile and invasive cells, it is not surprising that they have been widely used to study the cell intrinsic mechanisms underlying cancer cell invasion. By analysing melanoma cells under conditions resembling the 3D ECM, two major contributions to cellular invasion have been identified (Fig. 3). As such, cells can undergo cell-shape changes in order to adjust to the 3D-architecture of the tumour microenvironment and they can modulate the ECM through protease activities, such as matrix-metalloproteinases (MMPs) [2–5]. Considering these contributions, cells can invade in a mode that is regulated through integrin-mediated adhesion and is limited by their MMP activity, or a mode that uses cell-shape changes and is less restricted by protease activities [2,76]. The latter mode of invasion is represented by a round cell-shape that shows reduced substratum adhesion, and is associated with RHO-dependent and phospho-MLC driven actin cortex contractions and formation of membrane blebs [3,77].

What makes melanoma cells so efficient in invasion is the plasticity that is observed between the two modes of invasion described above (Fig. 3), as this allows the cells to perfectly adapt to the extracellular environment [4]. The switch between modes of invasion is regulated by the interplay of RHO GTPase exchange factors and GTPase activating proteins, whereby RHO activation leads to RAC inactivation and vice versa to control melanoma cell invasion plasticity [78]. Interestingly, while elongated cells use integrins and are absolutely dependent on MMPs to invade, also contractile cells express and secrete MMPs (MMP-13 and MMP-9) and their invasion has been shown to be regulated by both enzymatic and nonenzymatic protease signalling [79].

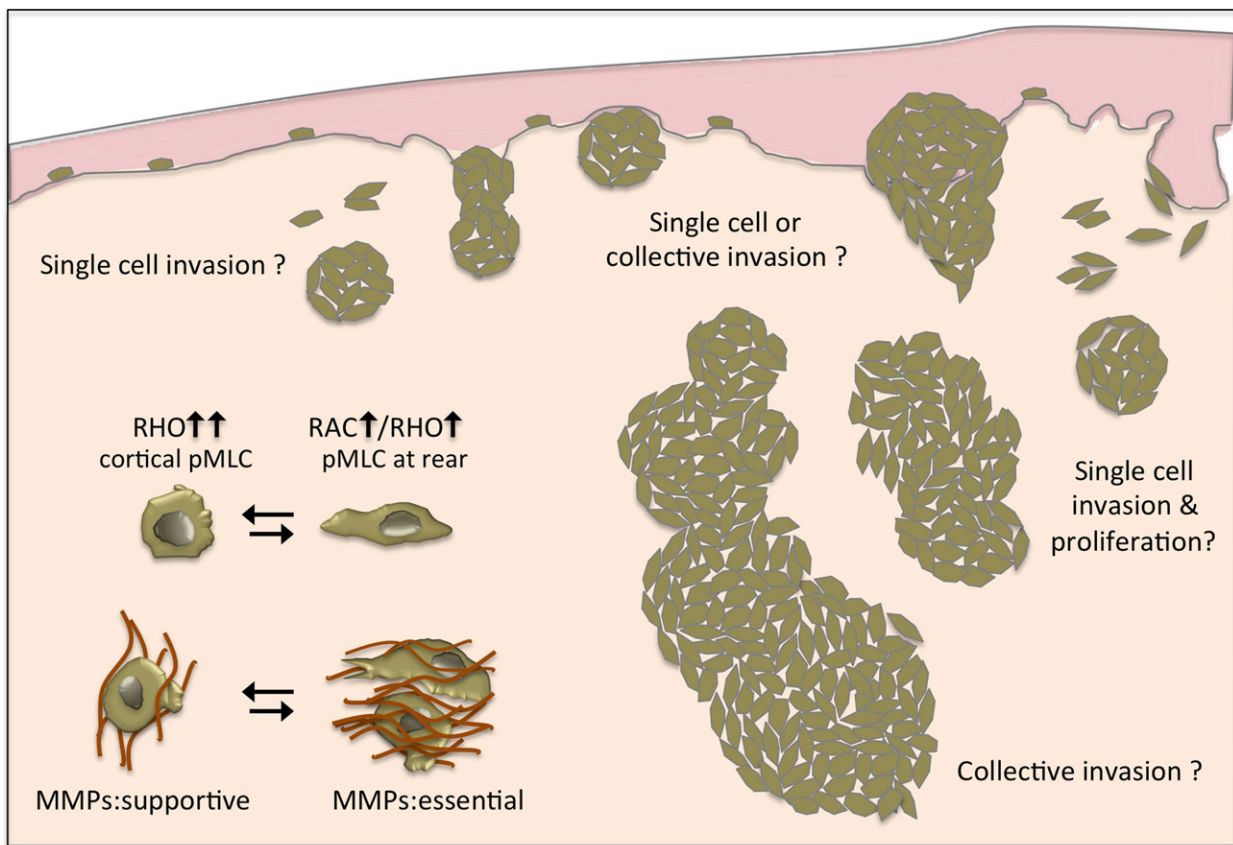
Differential signalling through RHO or RAC has also been linked to the overall mode of melanoma invasion, whereby the plasticity in RHO and RAC driven modes of invasion would support single cell invasion. Single cell invasion is considered a characteristic feature of melanoma cells as their nonepithelial nature suggests a low degree of cell-cell adhesion, allowing cells to invade ‘freely’ without contact to other cells. Single cell invasion can be observed in experimental settings *in vitro* and *in vivo* [27,62,78,80]. Moreover, while it is difficult to draw conclusions from the nondynamic situation found in human melanomas fixed for analysis, single cells can be seen even in histological sections of invasive melanoma ([www.proteinatlas.org](http://www.proteinatlas.org)).

What is often observed in melanoma biopsies, are groups of cells that have invaded the dermis (see Fig. 3), which could be the result of collective invasion. Collective invasion is typically detected in epithelial tumours, and occurs when cell–cell contacts are maintained and polarised migrating cells form a multicellular coordinated unit, which is driven by RAC-mediated activities [81]. As mentioned above, due to the nonepithelial nature of melanoma cells, instead of being the result of collective invasion, the observed invaded cell clusters could also be the product of some individual cells that started to proliferate after an initial invasive phase. Nevertheless, collective invasion appears to occur when cells break through the epidermal basement membrane and start invading the dermis (see Fig. 3).

### Phenotype switching and co-operativity

Regardless of whether single cell or collective activities are driving melanoma cell invasion, a question that is still left unanswered is whether melanoma cells have to change their phenotype in order to participate in invasion. Indeed, melanoma cells can undergo what is called ‘phenotype switching’, a process similar to ‘EMT’ that is observed with cells of epithelial origin [82]. In the concept of ‘phenotype switching’, MITF takes a central role as its abundant expression and the expression of many of its target genes (regulating cell cycle progression and pigmentation) is defining the so-called ‘proliferative phenotype’ [33]. The gene signature linked to the proliferative phenotype is void of genes that are linked to ‘invasion’ and ‘TGF $\beta$ ’ and hence describes a noninvasive phenotype [33]. As mentioned earlier, the downregulation of MITF is believed to initiate the ‘invasive phenotype’. In the ‘phenotype switching model’, cells switch between proliferative and invasive phenotypes throughout melanoma progression (Fig. 4), whereby the MITF-low/invasive phenotype performs invasion and dissemination, and cells switch back to the MITF-high/proliferative phenotype at the metastatic site in order to proliferate [82].

Intriguingly, using zebrafish transplantation assays, we made the striking observation that under heterogeneous conditions melanoma cells differing in their MITF expression levels (low and high) collaborated in their invasive behaviour, which allowed cells classified as noninvasive ‘proliferative phenotype’ to coinvasion with cells of the ‘invasive phenotype’ [80]. This concept, which we termed ‘co-operative invasion’ (Fig. 4), has been observed in other settings where individual cancer cells cooperate to drive tumour progression



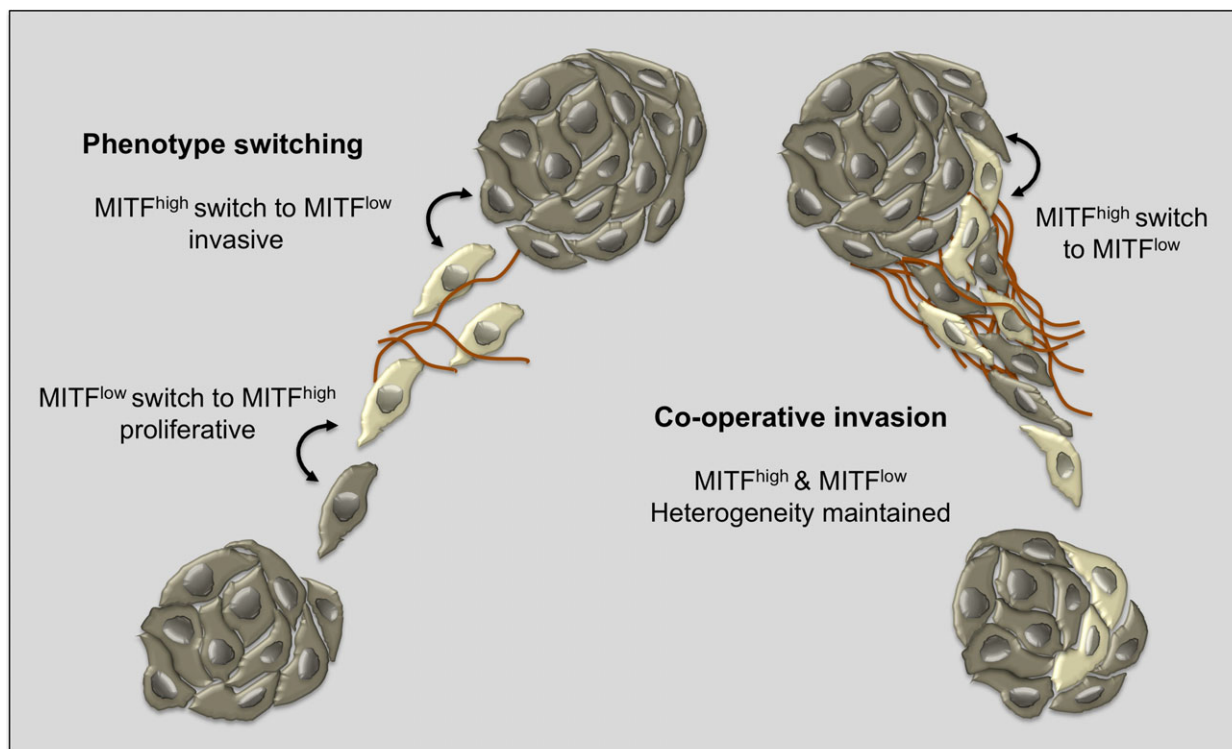
**Fig. 3.** Different modes of melanoma cell invasion. An illustration depicting various patterns of melanoma cell dermal invasion based on what can be seen in histological sections of melanoma lesions is shown. As these sections represent stills it is not clear whether the 'nests' of melanoma cells often seen in the dermis are the product of 'collective' invasion or of 'single cell' invasion followed by a proliferative phase. Clearly, 'single cell' invasion can occur as such cells can be detected in melanoma specimens. From experimental settings, we know that a more rounded shape of melanoma cells can be observed when cells possess high levels of RHO activity contracting cortical actin. In a dynamic process during invasion such cells can switch to a more elongated shape where high RHO activity is located at the rear of the cell, whereby invasion is driven by RAC activity at the front [4]. Elongated cells require MMP activity while invading by integrins, whereas rounded cells can use membrane blebs for invasion, but this is aided by the presence of MMPs [79].

[83–85]. The cooperation between individual cancer cells in a primary tumour might explain the observed heterogeneity in secondary tumours with regard to the mitotic, invasive and metastatic competence of distinct cell populations. In the context of invasion, we found that the invasive cells provided the noninvasive cells with MMPs, thereby allowing the proliferative phenotype to prevail [80]. Performing such co-operative behaviour suggests that no further switch is required, and when cells of the proliferative phenotype arrive at the secondary site they can proliferate. Importantly, we observe that when cells cooperate during invasion, reciprocal interactions alter the overall invasive behaviour of a tumour, which suggests that the current definitions of melanoma cell lines as invasive and noninvasive/proliferative are limited in the context of heterogeneity.

As we are only able to observe 'stills' of melanoma progression when analysing histological sections, it is impossible to state whether a metastasis is the result of 'phenotype switching' or 'co-operativity'. However, the fact that circulating melanoma clusters consisting of cells with high and low MITF expression have been isolated from patients [86], and the enormous phenotype heterogeneity that is observed in metastatic melanoma supports the idea that co-operativity occurs throughout melanoma progression.

### Invasion and therapeutic approaches

At present, the only clinical option to prevent the appearance of metastatic disease if melanoma is diagnosed early, is surgical resection of the primary tumours and/or lymphatic nodes and adjuvant



**Fig. 4.** Phenotype switching and co-operativity. Phenotype switching is thought to follow altered MITF expression in order to generate different phenotypes with only one phenotype being compatible with a particular stage of tumour progression [82]. During co-operative invasion, an initial switch creates an invasive cell, which then can cooperate with cells that have not undergone a switch to enable them to invade as well [80].

chemotherapy based on high dose interferon 2b, which has very little proven efficacy [87]. Despite the high metastatic potential of melanoma and the understanding of the molecular mechanisms governing melanoma cell invasion, only a handful of clinical trials have attempted to target this step of the metastatic cascade. Indeed, trials testing the multikinase inhibitor dasatinib failed due to excessive toxicity while the SRC specific inhibitor saracatinib provided no clinical responses [31,88]. In the same line, early phase trials assessing drugs targeting invasion through integrin signalling or MMP activity inhibition were not successful against advanced melanoma [89,90]. RAC inhibitors have been assessed in preclinical studies but not yet in clinical trials [91]. On the other hand, the central role played by the RHO/ROCK signalling in invasion and metastasis has led to the development and characterization of ROCK inhibitors as a mean to block melanoma progression, although it is yet to be defined whether the antitumor effect observed by ROCK inhibitors in mouse models of metastatic melanoma is uniquely due to its ability to inhibit contractile-dependent melanoma cell invasion or other biological

processes regulated by ROCK such as intra- or extravasation, cell cycle progression and/or cell viability [79,91–93].

### Invasion and MAPK pathway targeting therapy

In the last few years, the crosstalk between the molecular mechanisms governing melanoma cell invasion and resistance to targeted therapies against components of the MAPK pathway has gained growing attention. In melanoma, the *BRAF* protooncogene is mutated in around 44% of patients and BRAF and MEK inhibitors have been shown to produce profound but transient clinical responses [94,95]. In the majority of cases, patients relapse either due to reactivation of the MAPK pathway or through the induction of compensatory pathways, such as the PI3K/AKT cascade [96]. Furthermore, as a direct consequence of BRAF inhibition, increased activity of ROCK1 is observed, which appears to be due to reduced expression of RND3 when the MAPK pathway is inhibited [97,98]. Despite increased RHO/

ROCK activity, inhibitor treated cells display an elongated shape and display increased invasion, a phenomenon that is also observed when melanoma cells are treated with MEK inhibitors, where the increased invasion is dependent on integrins and MMPs [27,99]. Moreover, paradoxical activation of the MAPK pathway by BRAF inhibitors in NRAS mutant melanoma cells leads to increased invasion and metastatic potential, while BRAF mutant melanoma cells selected for their resistance to the BRAF inhibitor vemurafenib show increased invasion through reactivation of the MAPK pathway, again in a protease (and hence integrin) dependent mode of invasion [100]. It is therefore not surprising that the context of the increased invasion observed upon MAPK pathway, SRC kinase activation seems to play a key role. This observation has led to the proposal of combinatorial therapies based on both MAPK inhibitors and SRC inhibitors to tackle both invasion and growth [27,99,101]. Following this scientific rationale broad-spectrum, panRAF inhibitors that also show activity against SRC are being tested [102]. Interestingly, there might even be a crosstalk between actin cytoskeleton regulators and MAPK signalling with regard to melanoma cell survival as combination of ROCK inhibitors with either BRAF or MEK inhibitors enhances cell killing [98,103].

Among tumours progressing on MAPK inhibitors, ~ 50% show upregulation of receptor tyrosine kinases such as AXL or EGFR, and concomitant reduced expression of the transcription factor MITF [7,104,105], which leads to resistant tumours with a de-differentiated, invasive phenotype [7]. Enormous effort is put into understanding the drivers of this particular phenotype, and the idea of targeting receptor tyrosine kinase signalling is currently considered as therapeutic option. However, there is a clear need to better understand the signalling that is downstream of these receptors and that is linked to the 'invasive' signature found in these resistant tumours.

## Concluding remarks

Melanomas are highly metastatic skin tumours and metastatic disease is notoriously difficult to manage. As one of the first steps in the metastatic cascade, invasion has been the focus of intense research over the past 20 years, and melanoma has often been used as a model for cancer cells with invasive capacities. The molecular mechanisms governing melanoma cell invasion have unveiled how plastic these cells are depending on changes in the microenvironment, and the neural crest origin of melanocytes seems to be at

the bottom of the high capacity of melanoma cells to disseminate. While we have gained a good understanding of the molecular mechanisms by which melanoma cells invade, there are still important questions that remain unanswered.

It is generally believed that tackling invasion can prevent the development of lethal metastases, but a growing number of reports using preclinical models or describing the results of detailed gene expression analyses suggest a weak correlation between the phenotype of invasive cells and that of cells from metastatic sites. Mechanisms such as phenotype switching or co-operativity as metastatic strategy could explain these discrepancies, and therefore targeting these processes might be more appropriate, and we need to invest efforts into dissecting these mechanisms and identifying the key players.

What should not be forgotten is that from a clinical point of view the suitability of 'invasion' as the target for antimetastatic-oriented therapies, particularly for melanoma, is debateable. First, the plasticity of melanoma cells that enables them to adapt to new environments and to switch modes of invasion upon therapeutic intervention poses an intrinsic difficulty to stop invasion. Second, from a purely strategic point of view it has not yet been determined how important invading capacities are for the overall metastatic potential of melanoma cells. This is reflected for instance in the fact that beta-catenin mutations result in less invasion, yet a greater metastatic potential. Indeed, the metastatic cascade is a complex, multistep process that requires many other biological skills (such as adhesion to blood vessels, resistance to anoikis in the blood flow, proliferating activities). However, currently a major limitation to fully understand the relevance of invasive activities for the metastatic potential of melanoma cells is the fact that the majority of cell lines used in the melanoma community to study these processes are derived from lymph node or skin metastases, sites that do not necessarily reflect the lethal metastases of melanoma. Indeed, a thorough comparative study between primary tumours and distant metastatic lesions is lacking due to the scarcity of samples obtained from distant sites such as the brain, liver or lung. Even in the over 300 samples utilised in the TCGA study [106], only nine corresponded to distant organ metastases, the majority are from lymph node and skin metastases. Undertakings such as the 100 000 Genomes Project ([www.genomicsengland.co.uk](http://www.genomicsengland.co.uk)) aim to close this gap, which is urgently needed in order to enable us to target the properties that make melanoma cells so metastatic. Clearly, genes included in the 'invasive' signature are contributing to the 'aggressiveness' of melanoma as this situation is found in tumours

resistant to current therapies, so identifying these players might also help to improve the current standard of care.

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## Author contributions

Both authors discussed and developed the concept of the review and wrote the manuscript.

## Conflicts of interest

None.

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